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Neutrophil dysfunction, immature granulocytes and cell-free DNA are early biomarkers of sepsis in burn injured patients: A prospective observational cohort study.

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Short Title: Neutrophil dysfunction in post-burn sepsis

INTRODUCTION

Major burn injury results in a systemic inflammatory response syndrome (SIRS) characterised by an increase in circulating pro- and anti-inflammatory cytokines[1] and a simultaneous immunoparesis, increasing the susceptibility of patients to nosocomial infections and sepsis.[2] Whilst improvements in burn care have improved initial patient outcomes,[3] the prevalence of sepsis and its associated mortality remains significant.[4] The diagnosis of sepsis represents a major clinical challenge, as many classical diagnostic biomarkers are masked by the ongoing SIRS following major burn injury.[5] Numerous potential biomarkers of sepsis have been suggested including circulating levels of procalcitonin,[6] C-Reactive Protein[7] and interleukin 6.[8] However, these biomarkers lack specificity as they are also elevated during the initial SIRS response to sterile injury.[9-10] Understanding the mechanisms underlying the development and progression of sepsis is therefore critical if novel biomarkers for the accurate prediction and/or diagnosis of sepsis in burn-injured patients are to be discovered and novel therapeutic targets for its prevention and/or treatment identified.

Neutrophils provide frontline protection against rapidly dividing bacterial and fungal infections, common in burn-injured patients. Their antimicrobial functions include phagocytosis, the generation of toxic intracellular intermediates, and the ability to produce neutrophil extracellular traps (NETs).[11-12] Comprised of a DNA backbone decorated with granule-derived peptides, enzymes and modified histones, NETs have been shown to ensnare, and in some instances directly eliminate extracellular bacteria.[11, 13-14] In addition to their defensive role, modified histones and cell free DNA (cfDNA) are also potential biomarkers of sepsis,[15-16] having been identified in response to sterile injury and also during subsequent septic episodes following burn and traumatic injury.[16-18] However, whilst cfDNA is thought to originate predominantly from neutrophils, it is non-specific to the process of NET formation (NETosis) and can originate from a number of sources.[19] It has yet to be conclusively established whether NETosis occurs in patients following burn injury or could be used as a diagnostic indicator of sepsis in this setting.

Whilst impairments in neutrophil function have been proposed to underlie the increased incidence of nosocomial infections and sepsis after burn injury, few studies have investigated this relationship. Alexander *et al* showed that neutrophils from infected patients had impaired bactericidal activity compared to those from non-infected patients.[20] Similarly, Sheng *et al* demonstrated that the presence of sepsis was associated with a reduction in neutrophil bactericidal function.[21] More recently, it has been demonstrated that neutrophils isolated from patients with sepsis display a spontaneous migratory phenotype that is not present in patients with SIRS in the absence of sepsis.[22] Importantly, this phenotype was observed prior to the diagnosis of sepsis, thereby demonstrating the potential use of neutrophil functional analysis in predicting as well as diagnosing the development of sepsis.

To thoroughly characterise the longitudinal neutrophil response to burn injury and investigate its potential relationship with outcome, we measured peripheral blood neutrophil function and biomarkers of NETosis in a cohort of severely burn-injured patients. Patients were monitored for the development of sepsis and underwent serial sampling over one year post-injury. In addition, we studied the potential predictive capacity of three novel biomarkers of sepsis in burn injury; 1) immature granulocyte (IG) count, 2) neutrophil phagocytosis and 3) plasma cfDNA.

METHODS

Patients

63 consecutive patients admitted to the Queen Elizabeth Hospital Birmingham Burns Centre with a burn size of ≥15% total body surface area (TBSA) were recruited into a prospective cohort study within 24 hours of their injury (see Supplemental Figure 1 for CONSORT diagram). Patient demographics are shown in Table 1. In addition, healthy adult volunteers, who acted as the control cohort, were included in the study. Blood samples were collected into BD Vacutainers® (Becton Dickinson, Oxford, UK) containing either lithium heparin, EDTA, z-serum clotting activator or 1/10 volume of 3.2% trisodium citrate. Blood samples were collected at fixed intervals following injury (day 1 [< 24 hours post-injury], day 3 [+/- 1 day], day 7 [+/- 1 day], day 14 [+/- 3 days], day 21 [+/- 3 days], day 28 [+/- 3 days], month 2 [+/- 3 days], month 3 [+/- 7 days], month 6 [+/- 7 days] and month 12 [+/- 7 days]). Those patients that died during the study (n=20) and those patients that were lost to follow up were included in the analysis. A diagnosis of sepsis was made when at least 3 of the sepsis trigger criteria agreed in 2007 by the American Burn Association (ABA)[23] were met along with either a positive bacterial culture or when a clinical response to antibiotics was observed. Sepsis criteria were assessed on a daily basis. The source of sepsis for each episode was made through prospective recording of adverse events during the study. Pneumonia and ventilator associated pneumonia (VAP), urinary tract infection (UTI) and central line associated blood stream infection (CLABSI) were diagnosed according to the US Centres for Disease Control (CDC) criteria. The presence of multiple organ failure (MOF) was assessed daily using the Denver Post-injury MOF score and was defined as a score of >3 on two consecutive days with contribution from two organ systems.[24] The APACHE II score[25] and SOFA score[26] were also evaluated for the first 24hrs of admission. The abbreviated burn severity index (ABSI)[27] and the revised Baux score (rBaux)[28] were also calculated for each patient.

Measurement of neutrophil phagocytosis and oxidative burst

Using the commercially available PhagoTEST® and PhagoBURST® kits (BD Biosciences, Oxford UK), and following manufacturer's instructions, neutrophil phagocytosis of opsonised *Escherichia Coli* (*E.coli*) and ROS production in response to *E.coli* was measured in 100µl aliquots of heparinised whole blood. For both assays, 10,000 neutrophils, gated according to their forward scatter (FS)/sideward scatter (SS) properties were analysed on an Accuri C6 flow cytometer and data evaluated using CFlow software (BD Biosciences). Phagocytic Index was calculated by multiplying the percentage of cells able to phagocytose bacteria by the mean fluorescence intensity of the cells.

Analysis of blood cell distribution

Whole blood cell counts were performed on EDTA anticoagulated blood using the Sysmex XN-1000 haematology analyser (Sysmex UK, Milton Keynes, UK). The instrument also provides several novel automated fluorescent flow cytometric parameters including measurement of IG numbers. Quality control material (XN check) was tested on a daily basis to ensure instrument performance throughout the study.

Preparation of platelet free plasma (PFP)

Citrate anticoagulated blood was centrifuged at 2000 x g for 20 minutes at room temperature and the top 2/3rds of plasma carefully removed. Plasma was then centrifuged at 13,000 x g for 20 minutes and the top 2/3rds of the platelet free plasma removed and stored at -80°C.

Fluorometric analysis of plasma and serum cfDNA levels

cfDNA levels were measured by a fluorometric assay using SYTOX® Green Dye (Life Technologies, Cheshire, UK). 100 µl of supernatant from neutrophils stimulated with 25 nM phorbol myristate acetate (PMA) for 3 hours, or 10 µl of plasma was incubated with 5 µM SYTOX® Green Dye for 10

minutes and fluorescence was measured using a BioTek® Synergy 2 fluorometric plate reader (NorthStar Scientific Ltd, UK) with excitation and emission set at 485 nm and 528 nm respectively. For calibration of samples a λ-DNA (Fisher Scientific, UK) standard curve was utilised. The inter-assay and intra-assay coefficients of variation were 5.3% and 5.1% respectively.

Real-time quantitative PCR (qPCR) for the quantification of plasma nuclear DNA (ncDNA) and mitochondrial DNA (mtDNA) levels

DNA was isolated from 150 µl of PFP using a QIAamp DNA Blood Mini Kit (Qiagen) and eluted in 50 µl of nuclease-free water of which 5 µl was used in the PCR reaction. Plasma ncDNA and mtDNA were measured by qPCR using the SYBR Green 480 Probes Master kit (Roche) and analysed using a Light Cycler 480 (Roche). Primer sets used to amplify mtDNA and ncDNA were specific for the genes encoding cytochrome b (forward 5'-ATGACCCCAATACGCAAAAT-3' and reverse 5'-CGAAGTTTCATCATGCGGAG-3') and β-globin (forward 5'-GTGCACCTGACTCCTGAGGAGA-3' and reverse 5'-CCTTGATACCAACCTGCCCAG-3') respectively and were synthesized by Eurofins MWG. Primer sequences have no significant homology with DNA found in any bacterial species published on BLAST. For concentration determination, a standard curve was created using purified ncDNA or mtDNA, isolated from K562 cells. Samples that produced no PCR products after 40 cycles were considered undetectable and the Ct number set to 40 for statistical purposes.

Western blot protocol for detection of citrullinated histone H3

Citrullinated histone H3 in PFP and HL-60 positive control lysates was measured using SDS-PAGE and Western blotting. 1 µg/ml of primary antibody (ab5103, Abcam) to citrullinated histone H3 was used. Antigens were detected using Enhanced Chemiluminescence (GE Healthcare Life Sciences) and visualised using ChemiDoc™ Technology (BioRad, UK). To ensure equal loading, total protein was visualised using a Ponceau S Stain (G. Biosciences, USA).

Ex Vivo NET generation

Neutrophils were isolated from EDTA anticoagulated blood samples by Percoll density gradient centrifugation (Scientific Lab Supplies, UK). 2 x 10^5 neutrophils were stimulated with 25 nM PMA or 100 ng/ml lipopolysaccharide (LPS) (both from Sigma-Aldrich, Dorset UK) for 3 hours at 37^{o}C in a 5% CO_2 atmosphere. Following stimulation, samples were centrifuged at 2,200 x g for 10 minutes, after which cell-free supernatants were collected and immediately analysed for extracellular DNA content using the SYTOX® Green assay.

Visualisation of ex vivo NETosis by fluorescent microscopy

2 x 10^5 isolated neutrophils were seeded onto glass coverslips (VWR International) and stimulated with 25 nM PMA for 3 hours (37^{o}C and 5% CO_2 atmosphere). Following stimulation, cells were fixed with 4% paraformaldehyde (37^{o}C and 5% CO_2 atmosphere), permeabilised with 0.1% Triton X-100, and stained with 1 µM SYTOX® Green. Once stained, slides were mounted in fluoromount medium and visualised using a LEICA DMI 6000 B microscope at x 20 objective.

Statistics

Data were checked for normality using the Shapiro-Wilk test. Continuous variables were compared using a Mann-Whitney test or an unpaired T-test, with a Bonferroni correction for multiple comparisons. Categorical variables were compared using a Chi-squared test. Logistic regression analyses were conducted to examine the relationships between neutrophil function, cfDNA levels, and number of immature granulocytes at pre-specified sample times (e.g. day 7) and the presence of sepsis. Discriminatory power was assessed through the area under the receiver operator characteristic curve (AUROC). Longitudinal analyses were performed using linear mixed-effects models. Analysis was performed using the statistical software packages SPSS (IBM) and R version 3.0.1 (http://www.r-project.org) together with the lme4, effects, rms and pROC packages.

Study approval

Ethical approval for the study was granted by a UK NHS research ethics committee (Reference 12/EM/0432). Where possible written informed consent was received from participants prior to their inclusion in the study. Due to the severe nature of the injuries being studied, the ethics committee approved the use of a legal consultee, either personal or nominated, if the patient was not initially able to consent for inclusion in the study themselves. When the patient regained capacity, they were approached to give written consent to continue to participate in the study.

RESULTS

Circulating neutrophil and immature granulocyte count are elevated following burn injury

Relative to healthy volunteers, the circulating number of neutrophils following burn injury was significantly elevated ($p<0.005$) within 24 hours of injury (D1) (Figure 1A). Neutrophil count normalised at day 3 post-injury before becoming significantly elevated at day 7, and remaining elevated for 28 days post-injury. This was accompanied by an increase in both the number and frequency of circulating IGs, which were elevated within 24 hours of injury, returned to normal levels at day 3, before becoming elevated again at day 7 and remaining elevated for 28 days (Figure 1B and 1C). Morphological analysis of Giemsa stained isolated neutrophils confirmed the presence of immature cells with classical banded nuclear morphology (Figure 1D, arrows). The example shown is from a patient with a 45% TBSA burn during a septic episode at day 7 post-injury.

Neutrophil function is reduced following burn injury

Compared to the levels measured in healthy volunteers, neutrophil oxidative burst capacity was significantly reduced ($p<0.005$) in response to burn injury (Figure 2A). This reduced function was evident by day 3 post-injury and reached its lowest point 7 days after injury. In addition, there was a significant reduction in the phagocytic index (PI), which was evident within 24 hours of injury (Figure 2B). The reduction in oxidative burst capacity and PI both persisted, not returning to levels comparable to those of healthy volunteers until 2 months post injury. Interestingly, neutrophil PI on day 1 post-injury showed a showed a significant negative correlation with %TBSA ($r=-0.429$, $p=0.001$), % full thickness burn ($r=-0.337$, $p=0.01$), and presence of inhalation injury ($r=-0.327$, $p=0.12$) suggesting that neutrophil dysfunction is greater in those individuals with a more severe injury leaving the patient at increased risk of infection. To investigate whether burn injury affected NET production by circulating neutrophils, DNA release from isolated neutrophils stimulated with PMA was measured. Neutrophils from burn injured patients released lower levels of DNA compared to neutrophils isolated from healthy volunteers, and this was significant at days 3 and 7 post-injury

(Figure 2C). Fluorescence microscopy confirmed that neutrophils isolated from burn injured patients were partially resistant to NETosis induced by PMA (Figure 2D, right panel).

The release of IGs post-burn injury is associated with reduced neutrophil function

Flow cytometric analysis of neutrophils following burn injury revealed a group of patients who displayed a subset of neutrophils that exhibited greater forward-scatter and reduced sideward-scatter properties when compared to the 'normal' neutrophil population (Figure 3A). This population also exhibited reduced oxidative burst capacity and phagocytosis (Figure 3A and B). Whilst further phenotypic analysis of this population would be required to definitively identify these cells as IGs, the presence of this second population (termed 'dual population') showed similar kinetics to the emergence of IGs as measured using a haematology analyser (Figure 1B), being significantly elevated from day 3-28 post burn (Figure 3C). In addition, there was a significant correlation ($r=0.65$, $p<0.0001$) between the percentage of neutrophils that fell within the second population and IG frequency (Figure 3D). These data suggest that the release of IGs in to the circulation post burn injury is at least in part responsible for the reduction in neutrophil function.

Neutrophil function is reduced to a greater degree in septic patients

Neutrophil PI and oxidative burst capacity were compared between patients who had one or more septic episodes during their clinical course and those that had no septic episodes. Amongst the cohort of 63 patients, 6 died within 7 days of injury from non-septic causes and were removed from the analysis as it was not possible to determine if these patients would have developed sepsis. Of the 57 remaining patients, 35 exhibited one or more septic episodes during their clinical course representing a prevalence of 61%. The characteristics of the two groups are summarised in Table 1. Information regarding the timing and source of sepsis is displayed in Supplemental Table 1. Whilst oxidative burst capacity was reduced to a similar degree over the first 7 days post-burn, it remained reduced in the septic patients for a more prolonged period of time (Figure 4A). Figure 4A shows the

predicted fixed effects of time on oxidative burst capacity by sepsis status. The significant interaction term between sample day and sepsis (p=0.004) suggests that the relationship between sample day and oxidative burst differs by sepsis group. For neutrophil PI, the predicted fixed effects of time on PI by sepsis status was significant (p<0.001) suggesting the sepsis group have a lower ability to phagocytose bacteria (Figure 4B). We went on to compare IG levels between patients who had one or more septic episode during their clinical course with those who did not. We found elevated total neutrophil counts in patients who were septic or not, with no difference between the groups (Figure 4C). However, there were higher circulating IG levels in patients who developed sepsis compared to those that did not (Figure 4D). The difference between groups was at its greatest at 7–28 post injury and was statistically significant at days 7 and 14 (Supplemental Table 2).

Longitudinal analysis of plasma cfDNA levels following thermal injury

When measured using fluorometry, admission levels of total plasma cfDNA were not elevated compared to healthy volunteers, but were significantly elevated (p<0.005) at days 7 and 14 post-burn (Figure 5A). Interestingly, levels of cfDNA in admission serum samples were significantly higher (p=0.0001) than that found in matched plasma samples (Figure 5B), which may explain recent studies showing elevated admission levels of serum cfDNA following thermal injury.[29] Quantitative PCR analysis of plasma levels of nuclear DNA (ncDNA) and mitochondrial DNA (mtDNA) revealed that whilst there was no significant elevation of mtDNA following thermal injury, there was a significant elevation of ncDNA between day 1 and day 28 post-injury (Figure 5A). The significant elevation of ncDNA at day 1-3 post-injury is most likely due to the higher sensitivity of the PCR assay compared to the fluorometric assay (lower limit of detection; 0.1ng/ml vs. 4ng/ml). However, plasma ncDNA levels showed a similar pattern to cfDNA levels peaking at day 7 and 14, and there was a strong positive correlation between the two measurements (r=0.763, p<0.001). Longitudinal analysis showed that plasma cfDNA levels were elevated to a greater degree (p=0.049) in septic patients

compared to non-septic patients (Figure 5C). In non-septic patients, there was a minimal increase in plasma cfDNA, being similar to levels found in healthy volunteers (Figure 5C).

Neutrophil Extracellular Trap formation following thermal injury

Having found evidence of elevated levels of plasma cfDNA in septic patients, we investigated whether NETs could be a source of extracellular DNA. To confirm NETosis, plasma samples were analysed for the presence of Cit H3, which is a characteristic feature of NET generation. The results obtained for a representative patient with a septic episode are shown in Figure 5D (representative of n=9). Cit H3 was clearly detected in the plasma and coincided with the peak in cfDNA levels (Figure 5D). Cit H3 was not detected in any plasma sample obtained from non-septic patients or healthy volunteers (n = 10) (data not shown).

Potential use of IG number, phagocytic index and cfDNA as biomarkers of sepsis in burns

Having found differences in IG numbers, neutrophil function and circulating cfDNA levels between septic and non-septic patients, we examined the discriminatory ability of these variables to distinguish between the septic and non-septic cohort using the area under the receiver operating characteristic curve (AUROC). As these measurements have been suggested as potential novel biomarkers of sepsis in other studies, we tested their combined measurement for their discriminatory power at days 1, 3, 7 and 14 post-injury (Table 2). When two variables were used in the model, PI and IG count gave the best discriminatory power at day 1 with an AUROC of 0.921 and also showed moderate discriminatory power at day 3 (0.785). The combination of cfDNA and IG count showed good discriminatory power at day 1 (0.829), whereas cfDNA and PI showed good discriminatory power at day 1(0.815), 7 (0.826), and 14 (0.852). When these three variables were combined, they gave strong discriminatory power at day 1 (0.935) although with 24 cases observed in 33 patients, there is a risk of model over-fitting and the AUROC should be interpreted with caution. Finally, given the negative correlation found between % TBSA and phagocytic index, and %TBSA and presence of inhalation injury, we tested the discriminatory power of a combination of

rBaux score and the identified biomarkers (Supplemental Table 4). Importantly, the combination of rBaux score, Phagocytic index and IG count gave the highest AUROC (0.986) and was higher than any of these measurements alone. We were unable to test the combination of rBaux score, IG count, PI and cfDNA as the relatively low number of observations meant that the models were unreliable. The AUROC values for the individual variables are shown in Supplemental Table 3.

DISCUSSION

The diagnosis of sepsis in burn injured patients represents a major challenge as many of the diagnostic criteria for sepsis are present due to the ongoing SIRS response and are not specific for infection.[5] Moreover, a delay in diagnosis of only a few hours has shown to lead to increased mortality.[30] This led the Surviving Sepsis Campaign to publish guidelines in 2012 recommending the administration of intravenous antibiotics within the first hour of recognition of severe sepsis or septic shock[26]. Pathogen detection in blood cultures has been used as a gold standard for the diagnosis of sepsis in some studies, for example, in evaluating the performance of clinical diagnostic criteria.[31] This has limited clinical utility since 1) culture results are typically not available until 48 hours after sampling and 2) the majority of clinical studies report negative cultures in as many as 40% of severe sepsis patients.[32] Thus, the identification of novel biomarkers for the prediction and/or early diagnosis of sepsis are crucial. Here, we report that the combination of IG count, neutrophil phagocytic index and circulating cfDNA measurements shows good discriminatory power to predict later development of sepsis as early as day 1 post-injury. In addition, when we included the rBaux score to any combination of one or two of these parameters, the discriminatory power was improved even further for day 1 data. In particular, the [IG + PI + rBaux] model had the greatest discriminatory capacity with an AUROC of 0.986 (0.955, 1.000). These findings highlight the potential utility of a combination of clinical and novel immune biomarker data for the early prediction and/or diagnosis of sepsis.

We also found that neutrophil dysfunction was significantly prolonged in septic patients, suggesting that neutrophil dysfunction post burn may leave the patient susceptible to bacterial infection and consequent sepsis and thus may have both prognostic and diagnostic relevance for sepsis. In support of this, a recent study in a small cohort of burn injured patients demonstrated a spontaneous neutrophil migratory phenotype present only in cells isolated from those patients who developed sepsis. This phenotype was apparent 48 hours prior to the diagnosis of sepsis, and showed a good

predictive value.[22] The same group found that correcting burn-induced neutrophil dysfunction improved survival in a rodent model of sequential burn-injury and sepsis.[33]

Flow cytometry analysis of neutrophils post injury revealed the presence of a population of circulating neutrophils that had distinct forward scatter and sideward scatter properties when compared to the normal neutrophil population. The presence of these cells, which showed reduced functionality, coincided with the release of IGs, and the frequency of cells comprising the second population showed a significant positive correlation with IG numbers. Importantly, IG numbers were only significantly elevated across time in those patients who developed sepsis. Similar observations have been made in the intensive care unit setting. For example, Guérin and colleagues found that sepsis was associated with an increased frequency of circulating IGs. Moreover, IGs had good predictive value for sepsis deterioration 48 hours after admission.[34] Importantly, IG count has recently shown to be able to discriminate between patients with SIRS and sepsis, with a sensitivity of 89.2% and a specificity of 76.4%.[35] This is in agreement with our data, which showed that IG count could accurately discriminate between septic and non-septic patients with SIRS. This is of particular importance in burn-injured patients, where sepsis is challenging to diagnose as many of the diagnostic criteria are masked by the ongoing SIRS response which occurs in the vast majority of patients with a burn >15% TBSA.[5]

It has been suggested that plasma cfDNA is a potential novel biomarker of sepsis. Longitudinal analysis of plasma cfDNA levels in our cohort of septic burn-injured patients revealed a potential diagnostic use for this biomarker. Plasma cfDNA levels following thermal injury were significantly higher in those patients who developed sepsis. cfDNA levels were maximally elevated during septic episodes and cleared effectively upon recovery of the patient. This is in agreement with other studies thus highlighting the potential of cfDNA as a novel biomarker for sepsis.[18, 24, 31] In addition, plasma cfDNA levels measured on the day of injury were able to discriminate between septic and

non-septic patients and this discriminatory power was improved further when compared with PI and IG count.

Circulating cfDNA is non-specific to NETosis and can also be released from apoptotic or necrotic cells as well as bacteria.[19] In order to provide conclusive evidence for *in vivo* NETosis, we analysed patient plasma for the presence of Cit H3. High levels of Cit H3 coincided with the maximal levels of cfDNA, demonstrating that NETosis is occurring during septic episodes and thus contributing to the increase in plasma cfDNA. These data agree with the work of Hirose *et al* who showed the presence of circulating Cit H3 only in those patients who were infected at the time of sampling.[16] Initially this observation appears to contradict our *ex vivo* data. However, there are a number of possible explanations for NET markers detected *in vivo* and reduced *ex vivo* NETosis. One possible explanation is that functional neutrophils have migrated and are generating NETs in the damaged tissues and thus leaving non-functional neutrophils in the circulation. Another possibility is that the high numbers of dysfunctional IGs released from the bone marrow are contributing to the reduced neutrophil function. Further studies are required to fully understand the mechanisms surrounding *in vivo* NET generation and the reduced *ex vivo* neutrophil function.

To summarise, we present a novel composite clinical-pathological biomarker model that may have predictive and diagnostic utility for post-burn sepsis, a devastating complication of severe burn injury. Our data also highlights that, in addition to being a potential diagnostic biomarker of sepsis, burn-induced neutrophil dysregulation is a potential therapeutic target, as correcting aberrant function may reduce susceptibility to later nosocomial infections and sepsis. Indeed, a recent study in a rat model has highlighted the potential of this therapeutic avenue.[33]

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Figure 1. Neutrophil and Immature Granulocyte (IG) numbers are elevated in the circulation following burn injury. (A) Circulating neutrophil count (log scale) (n=33), (B) circulating IG count (log scale) (n=33), and (C) % IGs across time following burn injury (log scale) (n=33). (D) Giemsa stain of peripheral blood neutrophils following burn injury. Arrows identify cells with immature banded nuclear morphology. Difference in cell counts at each timepoint were compared to healthy control (HC) (n=13) values using a Mann-Whitney test; *p<0.005.

Figure 2. Burn injury results in a prolonged neutrophil dysfunction. (A) Neutrophil oxidative burst capacity (n=63), (B) Phagocytic Index (n=63) and (C) ex vivo NET generation across time following burn injury (n=24). Data at each timepoint were compared to healthy control (HC) values using a Mann-Whitney test; *p<0.005. (D) Ex vivo NET generation in response to PMA by neutrophils isolated from a healthy control or a burn patient. Slides were mounted in fluoromount medium and visualised using a LEICA DMI 6000 B microscope at X20 objective.

Figure 3. Burn injury leads to the release of Immature Granulocytes (IGs) into the circulation. (A) Presence of a neutrophil “dual population” following burn injury. Neutrophils in the dual population show reduced phagocytosis and oxidative burst capacity. (B) Neutrophil function in patients with a dual population (yes) compared to patients without a dual population (no) (n=63). Groups were compared using a Mann-Whitney test; *p<0.05. (C) % of cells that comprise the dual population across time following burn injury (log scale) (n=33). Data at each time-point were compared to healthy control (HC) (n=15) values using a Mann-Whitney test; *p<0.005. (D) Correlation between % of cells in the dual population and IG count (log scale) (n=33).

Figure 4. Neutrophil dysfunction and elevated IG count is sustained in septic patients. Longitudinal analyses were performed using linear mixed-effects models to examine the relationship between time and (A,B) neutrophil function (n=57), (C) neutrophil count (n=33) and (D) IG count (n=33), according to sepsis status. Line represents predicted mean fixed effects, shaded area represents 95% confidence intervals.

Figure 5. Evidence of circulating NETs in septic burn injured patients. (A) Plasma cfDNA levels (log scale), mtDNA levels (log scale) and ncDNA levels (log scale) across time following burn injury (n=50). Data at each timepoint was compared to healthy control (HC) (n=10) values using a Mann-Whitney test; *p<0.005. (B) Levels of cfDNA in admission plasma and matched serum samples (n=17). Data were compared using a paired t-test; ***p=0.0001. (C) Longitudinal analyses were performed using linear mixed-effects models to examine the relationship between time and cell-free DNA levels according to sepsis status (n=50). Line represents mean predicted fixed effects, shaded area represents 95% confidence intervals. (D) Cell-free DNA levels and citrullinated histone H3 levels in the plasma of a representative septic patient across time following burn injury.